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SHORT COMMUNICATION

Genotoxicity of copper suplementation in cattle to prevent hypocuprosis

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RESUMEN

El cobre es un micronutriente esencial en bovinos. Usualmente la enfermedad más común asociada a este mineral es la hipocuprosis. Para prevenirla se indica la suplementación con cobre. Estudios previos demostraron la existencia de un efecto genotóxico dependiente de la deficiencia de cobre mientras que paralelamente se observó un efecto genotóxico asociado con la suplementación con Cu. El objetivo de este trabajo fue reportar la existencia de daño en el ADN, medido con el ensayo cometa, asociado a la suplementación con Cu. Se obtuvieron 22 muestras de sangre de animales normocuprémicos (grupo A) y de 20 animales normocuprémicos previamente suplementados con Cu (grupo B). Se determinó la concentración de Cu plasmático mediante espectofotometría de absorción atómica de llama y el daño en el ADN mediante el ensayo cometa versión alcalina. La concentración de Cu plasmático fue de 66,3 ± 5,9 µg/dl y 72,2 ± 8,6 µg/dl en los grupos A y B respectivamente. Con el ensayo cometa se observó un incremento significativo de núcleos con migración del ADN en el grupo B (49,6 ± 8,6) respecto del grupo A (29,0 ± 8,4) ($p<0,01$). El grado de daño en el ADN mostró 70,6 % ± 8,39 de células grado 1 en el grupo A y 50,4 % ± 8,58 de células grado 1 en el grupo B respectivamente ($p<0,01$). Para las células de grado 2 los valores fueron 19,5 % ± 7,45 y 41,0 % ± 9,5 para ambos grupos respectivamente ($p<0,01$). Los resultados obtenidos muestran un aumento significativo en el nivel de daño en el ADN de bovinos suplementados, probablemente asociado al efecto prooxidativo del Cu. Próximos estudios deberían encaminarse a determinar el origen de este daño, así como las consecuencias de la suplementación parenteral de los animales de granja.
Palabras clave: Genotoxicidad, suplementación con cobre, hipocuprosis bovina.

ABSTRACT

Copper is an essential micronutrient in cattle. Usually, the most common disease associated with copper is hypocuprosis. To prevent the copper deficiency, copper supplementation is indicated. Preliminary studies reported genotoxic effects of copper deficiency. Simultaneously, a genotoxic effect of copper supplementation was detected. The aim of this work was to report the DNA damage, assessed by comet assay, associated with copper supplementation in Aberdeen Angus cattle. Blood samples were obtained from 22 normocupremic Aberdeen Angus cows (group A) and from 20 Aberdeen Angus cows previously supplemented with parentheral copper (group B). Copper plasma concentration was determined by flame atomic absortion spectophotometry and DNA damage was assessed by alkaline comet assay. Copper plasma level were 66.3 ± 5.9 µg/dl and 72.2 ± 8.6 µg/dl in group A and group B respectively. Comet assay showed a significant increase of nuclei with DNA migration in group B (49.6 % ± 8.6) when it was compared with group A (29.0 % ± 8.4) ($p<0.01$). The degree of DNA damage showed 70.6 % ± 8.39 degree 1 cells in group A and 50.4 % ± 8.58 degree 1 cells in group B ($p<0.01$). For degree 2 cells, the percentages were 19.5 % ± 7.45 and 4.10% ± 9.5 for groups A and B respectively ($p<0.01$). Results obtained showed a significant increase of DNA damage in normocupremic animals with copper supplementation, probably associated with the prooxidant effect of copper. Further studies could contribute to elucidate the mechanisms involved in the induction of DNA damage, as well as the effect of copper supplementation in farm animals.
Keywords: Genotoxicity, copper suplementation, cattle hypocuprosis

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INTRODUCTION

Copper is an essential factor for development, growth and reproduction in cattle (McDowell, 1992). Usually, the most common disease associated with copper is hypocuprosis (copper deficiency). This disease is recognized as the second most widespread mineral deficiency that affects grazing cattle around the world (Ramirez et al., 1996). To prevent hypocuprosis copper supplementation is indicated (Underwood, 1981). However, discontinuous supplementation with soluble-complex, such as copper-calcium ethylene diaminetetra-acetic acid (Cu-Ca EDTA), implies a high risk of acute toxicity. This is so because copper is rapidly traslocated from the injection site to the liver (Underwood and Suttle, 1999) and acute copper toxicity depends on the rate of transfer from the injection site to the liver, rather than the total amount of copper injected (Giuliodori et al, 1997). Preliminary studies to detect genotoxic effect of copper deficiency showed a negative correlation between cupremia levels and DNA damage assessed by Single Cell Electrophoresis (Comet assay) (Picco et al., 2001), which is a rapid and sensitive technique to detect single and double strand breaks (SSB and DSB) as well as alkali labile sites (Rojas et al., 1999). However, statistical significant differences were found between normocupremic animals and cows with normal cupremia levels resulting from copper supplementation. Consequently, a study was carried out to analyze the possible genotoxic effect of copper supplementation. The aim of this work was to report the DNA damage, assessed by comet assay, associated with copper supplementation in Aberdeem Angus cattle.

MATERIAL AND METHODS

Blood samples were obtained in heparinized Vacuttainers®tubes (Franklin Lakes, NJ, USA) from 22 normocupremic Aberdeen Angus cows from pastures without copper deficiency (group A) and 20 from Aberdeen Angus cows from copper-deficient pastures which had been supplemented with copper one month before sampling. Supplementation was made with Cu-Ca EDTA subcutaneously injected (group B). This compound is a water soluble salt which has a rapid translocation to the liver used in 100-240 mg total doses (Underwood and Suttle, 1999). Each blood sample was divided into two aliquots, one for the comet assay and the other for copper plasma determination. For copper plasma determination blood samples were centrifuged and plasma was treated with 10% w/v thriochloacetic acid, separating the supernantant for Cu analysis. Copper concentration was analyzed by flame atomic absortion spectrophotometry (Piper and Higgins , 1967) using internal quality control. For the comet assay, samples were stored in darkness at 4° C for no more than 30 minutes. Comet assay was performed according to Singh et al. (1988) with some minor modifications. Fifteen ml of blood were mixed with 75 ml of low melting agarose 0.5% (Gibco BRL, NY, USA), seeded on a slide coated with 0.5% normal melting agarose (Promega, USA) and cooled until solidification. Two slides per animal were made. After this, the cells were lysed in a detergent solution (100 mM EDTA, 2.5 M NaCl, 10 nM Tris, 1% Triton X-100 and 10% DMSO) for at least 1 hour and stored until electrophoresis.

Before electrophoresis, the slides were equilibrated in alakaline electrophoresis solution (1mM EDTA, 300 nM NaOH, pH > 13) for 20 minutes. Electrophoresis was carried out for 30 minutes at 25 V and 300 mA (1.25 V/cm). After this, slides were neutralized by washing three times with Tris buffer (pH 7.5) every 5 min and distilled water.

Slides were stained with 1/1000, SYBR Green I (Molecular Probes, Eugene, Oregon, USA) solution (Ward and Marples, 2000) and the analysis was carried out using Olympus BX 40 microscope provided with a 100 W high pressure mercury lamp USHIO USH 102 D. Images were captured wit a Sony CCD camera and saved using the Image Pro Plus® software.

Blood leukocytes were first classified as normal (nuclei without DNA migration) or abnormal (nuclei with DNA migration). Further classification of abnormal according to Kobayashi et al. (1995) is shown in table II.

For the Statistical analysis the Statgraphics® PLUS ver. 3 software was used. Nuclei with or without DNA migration and comet degree in both groups were compared by the Students "t" test.

RESULTS

Table I summarizes the plasma levels of copper and the DNA damage measured by the comet assay according to the criteria of differentiated nuclei with or without DNA migration. In normocupremic cows the copper plasma concentration mean was 66.3 ± 5.9 µg/dl, while in supplemented animals the copper plasma concentration was 76.2 ± 10.8 µg/dl. The mean of nuclei without DNA migration was 71.0 % ± 8.4 in group A and 50.4 % ± 8.6 in group B respectively. Comet assay analysis showed significant differences between groups A and B ($p < 0.01$). Table II

summarizes the results obtained from comet assay, according to Kobayashi et al. (1995). The increase of DNA damage in group B was mostly evidenced by the decrease of comet degree 1 cells, from average of 70.6 % to 50.4 % in group A ($p < 0.01$) and an increase of comet degree 2, from an average of 19.5% to 41% ($p < 0.01$). Comparison of comet degree 3 cells between both groups no statistical differences were found ($p < 0.5$). Finally, in comet degrees 4 and 5 an increase of damage was found in group A ($p < 0.03$ and 0.02 respectively).

Table I.
Cupremia levels average and percentage of cells without DNA migration measured by the comet assay.

	Number of animals	Cu plasma Means	Cells without DNA migration	Cells without DNA migration
Group A	22	66.3 (± 5.9)	71 (± 8.4)	29 (± 8.4)
Group B	20	76.2 (± 10.8)	50.5 (± 8.6)	49.6 (± 8.6)

Table II.
Cupremia levels average and DNA damage levels according with Kobayashi , et al., (1995).

	Cu plasma Mean	Degree 1 %	Degree 2 %	Degree 3 %	Degree 4 %	Degree 5 %
Group A	66.3 (± 5.9)	70.6 (± 8.39)	19.5 (± 7.45)	6.4 (± 4.63)	2.6 (± 2.77)	0.8 (± 0.99)
Group B	76.2 (± 10.8)	50.4 (± 8.58)	41 (± 4.5)	5.9 (± 4.1)	1.3 (± 1.4)	0.2 (± 0.6)

DISCUSSION

Different sources of copper are available for subcutaneous injection: copper-calcium ethylene diaminetetra-acetic acid, copper-glycine, copper-hydroxiquinoline, copper sulphonate, and copper-heptonate, that belong to more water soluble compounds and cause lower tissue reaction in the injection point. However, they are rapidly translocated from the injection site to the liver, with a higher risk of acute toxicity. On the other hand, Methionate complexes are less toxic because of their lower translocation rate they but cause severe reaction at the site of injection, where part of the dose can be encapsulated (Suttle, 1981 a -b). Acute copper toxicity in cattle when given excessive amounts of copper soluble-salts, such as Cu-Ca EDTA derived in liver damage. This damage depends on the transfer rate from the injection site to the liver, rather than on the total amount of copper injected (Giuliodori et al., 1997). In the same way, copper plasma levels, as a function of liver copper store, could be independent of the copper supplementation source.

During several years different classifications, using copper plasma level, have been used to establish the animal copper status. In this way, one of the most accepted classifications, proposed by Suttle (1983), classifies the animals in severe hypocupremics (< 30 µg/dl), moderate hypocupremics (30-59 µg/ml), and normocupremic animals (60-120 µg/dl). Recently, Kincaid (1999) suggested a new value for normocupremic animals (70-120µg/dl). In both cases, the plasma copper concentration is used as an indicator. However, the possibility that normocupremic condition due to a copper supplementation was not taken into account.

Results obtained with the comet assay showed a statistical difference between normocupremic animals and animals from copper reaching normocupremic levels after copper supplementation. These results suggest a genotoxic effect associated with copper supplementation. Taking into account the prooxidant effect of copper (Proshaska, 1997), the increase of reactive oxygen species could be responsible for the increase of DNA damage, measured by the comet assay.

Cupremia fails to differenciate between supplemented and non supplemented normocupremic animals. Therefore, the genotoxic effect could also depend on the copper plasma supplementation source.

Further studies could contribute to elucidate the mechanism involved in the induction of DNA damage, its consequences and the importance of copper supplementation source in farm animals.

REFERENCES

- Giuliodori, M.J., Ramírez, C.E., Ayala, M. (1997). Acute copper intoxication after Cu-Ca EDTA injection in rats. Toxicology 124: 173-177.
- Kincaid, R.I. (1999). Assessment of trace mineral status of ruminants: A review. Proceedings of the American Society of Animal Science, 1999.
- Kobayashi, H., Sugiyama, C., Morikawa, M., Hayashi, M., Sofuni, T. (1995) A comparison between manual microscopic analysis and computerized image analysis analysis in the single cell gel electrophoresis assay. MMS Commun. 3, 103-115.
- McDowell (1992). Minerals in animal and human nutrition. Academic press, Inc. San Diego, USA.
- Picco, S.J., De Luca, J.C., Mattioli, G.A. & Dulout, F.N. (2001). DNA damage induced by copper deficiency in cattle assessed by the Comet assay. Mutation Research 498, 1-6.

- Piper, H.G. & Higgins, G. (1967). Estimation of trace metals in biological material by atomic absorption spectrophotometry. Proc. Assoc. Clin. Biochem. 7, 190-195.
- Prohaska, J.R. (1997). Neurochemical roles of copper as antioxidant or Prooxidant. Metals and oxidative damage in neurological disorders, edited by Connor. Plenum Press, New York, USA.
- Rojas, E., López, M.C. & Valverde, M. (1999). Single cell gel electrophoresis assay: methodology and application. J. Chromatogr. 722, 225-254.
- Singh, N.P., McCoy, M.T., Tice, R.R. & Schneider, E.L. (1988). A simple technique for quantitation of low levels of DNA damage in individual cells. Exp. Cell Res. 175, 184-191.
- Suttle, N.F. (1983). The nutritional basis for trace elements deficiencies in ruminant livestock. In Trace elements in Animal Production and Veterinary Practice, OCC Publication no. 7. Eds. Suttle, N.F., Gunn, R.G., Allen, W.M., Linklater, K.A. & Wiener, G. British Society of Animal Production.
- Suttle, N.F. (1981 a). Effectiveness of orally administrated cupric oxide needles in alleviating hypocupraemia in sheep and cattle. Veterinary Record 108, 417-420.
- Suttle, N.F. (1981b). Comparison between parenterally administrated copper complexes of their ability to alleviate hypocupraemia in sheep and cattle. Veterinary Record 109, 304-307.
- Underwood, E.J. (1981). Copper, molibdenum and sulfur. The mineral nutrition of livestock (2 ed.) Edit: Commonwealth Agric. Bureaux. Chapter 9: 91-111.
- Underwood, E.J. and Suttle, N.F. (1999). The mineral nutrition of livestock, 3rd Edition. CABI Publishing, New York, USA.
- Ward, T.H. & Marples, B. (2000). SYBR Green I and the improved sensitivity of the single-cell electrophoresis assay. Int. J. Radiat. Biol. 76, 61-65.